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(54) IMMUNOASSAY METHOD FOR BNP  
IMMUNOASSAY ZUM BESTIMMEN VON BNP  
PROCEDE DE DOSAGE IMMUNOLOGIQUE POUR BNP

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JP-A-3 297 392 JP-T-7 507 210

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• HUNT P J ET AL: "THE ROLE OF THE  
CIRCULATION IN PROCESSING PRO-BRAIN  
NATRIURETIC PEPTIDE (PROBNP) TO  
AMINO-TERMINAL BNP AND BNP-32"  
PEPTIDES, ELSMFORD, US, vol. 18, no. 10, 1997,  
pages 1475-1481, XP000913575 ISSN: 0196-9781  
• FEBS LETTERS, Vol. 400, No. 2, (1997), p. 177-182,  
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## Description

## TECHNICAL FIELD

**[0001]** The present invention relates to an immunoassay for the brain natriuretic peptide (BNP) which is a member of natriuretic peptide family, more specifically, it relates to an immunoassay for  $\gamma$ -BNP and derivatives thereof.

## BACKGROUND ART

**[0002]** Natriuretic peptide family includes three members, i.e., atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and type C natriuretic peptide (CNP). Among them, ANP and BNP are cardiac hormones which are mainly biosynthesized in and secreted from the heart. ANP and BNP are similar in structure. ANP is a peptide of 28 amino acids with a ring (circular) structure formed by a disulfide bond between the 7th and the 23rd cysteine residues, while BNP is a peptide of 32 amino acids with a ring structure formed by a disulfide bond between the 10th and the 26th cysteine residues. These mature peptides of 28 and 32 amino acids have been considered to be produced from respective precursor when a leader sequence is cleaved off intracellularly or at the time of secretion. That is, there has been reported that human BNP is first synthesized as a prohormone (hereinafter, referred to as pro-BNP) in myocardial cells, which is split before or at the time of secretion between Ser<sup>26</sup>-His<sup>27</sup> to give pro-BNP (hereinafter, referred to as  $\gamma$ -BNP), and which is further split between Arg<sup>102</sup>-Ser<sup>103</sup> to give BNP-32 (hereinafter, referred to as  $\alpha$ -BNP) and BNP(1-76), and that the former exhibits the activity. It has been considered that at least the ring structure must be remained for the expression of activity.

**[0003]** The secretion of cardiac hormones being stimulated by various heart diseases, it well reflects the change in the cardiac functions. The secretion of ANP is accelerated mainly when the atrium undergoes a load, while the biosynthesis and secretion of BNP are stimulated when the ventricle undergoes a load. Accordingly, both ANP and BNP are useful as indicators in the diagnosis of heart disease. As the progress of investigation in the *in vivo* role of respective hormone, the advantageous features of BNP as an indicator for diagnosing heart disease have become clear. For example, the blood concentration of BNP is only 1/6 of ANP in a normal subject but it becomes higher than ANP in patients of heart failure or the like; the blood concentration of BNP increases in the case of heart failure like ANP, and the plasma concentration of BNP often exceeds that of ANP reflecting more accurately the severity of heart dysfunction; the plasma concentration of both ANP and BNP elevates in peripheral blood and elevation rate is higher in BNP. Moreover, BNP level in patients of heart failure sometimes increases to several tens times to several hundreds times of that of healthy normal subjects, and the change of BNP in the cases of heart failure is so marked that no other hormones are incomparable therewith. For these reasons, the usefulness of BNP measurement has been suggested (Y. Saito et al., *Meibo*, 12(5), 28, 1995).

**[0004]** Under the conditions, an immunoassay which utilizes anti-BNP antibody and is applicable to the diagnosis of cardiac insufficiency has been proposed. Japanese Patent Publication (KOHYO) 7-507210 describes a method of measuring  $\gamma$ -BNP (1-76) produced by biodegradation by protease or the like. However, this method is directed to  $\gamma$ -BNP (1-76) which lacks the portion(s) essential for the expression of activity such as ring structure and, therefore, cannot determine the hormone activity directly.

**[0005]** An assay kit for the measurement of  $\alpha$ -BNP having natriuretic activity has been marketed ("BNP-32", Peninsula). With this kit, degradation products of  $\alpha$ -BNP in blood including fragments lacking activity due to the deletion of C-terminal region can also be measured. Taking the low blood concentration of BNP into consideration, the measurements involving the degradation products cannot be disregarded. Accordingly, the said method connotes disadvantages to be an assay for BNP in the establishment of an accurate diagnosis of heart failure.

**[0006]** As a kit for the measurement of BNP free from the disadvantages above has been marketed ("SHIONORIA BNP", Shionogi), which characteristically uses an antibody recognizing the structure essential for the expression of activity. However, this method would be affected significantly by the process for collecting and storing blood sample, because  $\alpha$ -BNP is extremely instable in collected blood. It is, therefore, suggested that the sample should be specifically treated by, for instance, adding an agent for inhibiting degradation into a blood collecting tube or maintaining the sample at low temperature so as to obtain reliable data. Such procedures may hamper the extensive clinical application of the said BNP assay kit.

**[0007]** In Hunt et al. (1997), *Peptides*, Vol. 18, pages 1475-1481, antibodies binding to the N-terminal part of pro-BNP and antibodies binding to BNP-32 are disclosed. However, the combined use of these antibodies in an immunoassay specific for pro-BNP is not disclosed.

## DISCLOSURE OF INVENTION

**[0008]** The present inventors have conducted research intensively for the purpose of establishing an accurate method

of diagnosing cardiac diseases involving BNP and found that BNP exists in blood in the form of  $\gamma$ -BNP or its degradation product which at least comprises structurally the  $\alpha$ -BNP moiety (hereinafter, they are referred to as " $\gamma$ -BNP derivative"), and not in the form of  $\alpha$ -BNP which has so far been considered to be dominant. The inventors have also found that  $\gamma$ -BNP is more stable than  $\alpha$ -BNP in blood, that is, one role of the N-terminal structure of  $\gamma$ -BNP, among many, would be the stabilization of BNP. The above indicates that an organism biosynthesizes at least 2 kinds of BNP molecule which share the BNP activity but differ in half-life. These findings led the present inventors to have a view that it is indispensable to establish a method specific for not only  $\alpha$ -BNP but also  $\gamma$ -BNP to accomplish an accurate diagnosis of cardiac diseases.

[0009] The present invention provides an immunoassay specific for mammalian  $\gamma$ -BNP derivatives, characterized in that it uses the first antibody which is reactive with mammalian  $\alpha$ -BNP and the second antibody which is reactive with mammalian prepro-BNP or  $\gamma$ -BNP derivatives and not reactive with  $\alpha$ -BNP.

[0010] As used herein, the term "mammalian  $\alpha$ -BNP" refers to a peptide of low molecular weight having natriuretic activity which is derived from mammalian prepro-BNP or  $\gamma$ -BNP through the removal of N-terminal region as a result of processing at the carboxy terminus of processing signal sequence. In case of human BNP,  $\alpha$ -BNP is a peptide consisting of C-terminal 32 amino acids (Nos. 103-134) of the amino acid sequence of SEQ ID NO: 1 and having  $\alpha$  ring structure. The carboxy terminus of processing signal sequence on the prepro-BNP molecule varies slightly depending on the species. For example, it is No. 102 Arg in case of human BNP while it is No. 100 amino acid in case of porcine or canine BNP.

[0011] As used herein, the term "mammalian  $\gamma$ -BNP" refers to a pro-BNP comprising a partial peptide of 32 amino acids corresponding to  $\alpha$ -BNP at the carboxy terminal region. In case of human  $\gamma$ -BNP, it is pro-BNP of 108 amino acids from No. 27 His to No. 134 His of the amino acid sequence of SEQ ID NO: 1. The term "prepro-BNP" refers to a peptide of 134 amino acids from No. 1 Met to No. 134 His of the amino acid sequence of SEQ ID NO: 1 in case of human.

[0012] As used herein, the term "mammalian  $\gamma$ -BNP derivative" refers to a peptide fragment derived from mammalian prepro-BNP or  $\gamma$ -BNP through mainly the *in vivo* protease reaction, which fragment includes or is larger than  $\alpha$ -BNP. Although  $\gamma$ -BNP derivative would comprise a molecule of the same or smaller size compared to  $\gamma$ -BNP in general, it may comprise a molecule larger than  $\gamma$ -BNP.

[0013] Otherwise specifically mentioned, as used herein, the term " $\gamma$ -BNP derivative" includes  $\gamma$ -BNP itself.

[0014] The term "stable", when used herein in connection with BNP, means that a BNP molecule maintains the C-terminal ring structure including C-terminus of BNP and the natriuretic activity after undergoing the degradation by protease, and that the said activity is not significantly decreased even 24 hours from the collection of blood samples. In light of this definition, the  $\gamma$ -BNP derivative as the target substance (analyte) of the present immunoassay is stable.

[0015] On the other hand, the term "unstable" means that a BNP sample undergoes degeneration by protease at the C-terminal region and that the natriuretic activity is significantly decreases 24 hours from the collection of blood samples. In light of this definition,  $\alpha$ -BNP is unstable.

#### BRIEF DESCRIPTION OF DRAWINGS

##### [0016]

Fig. 1 is a chromatogram obtained in an  $\alpha$ -BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample. In Fig. 1, A indicates the position of elution of  $\alpha$ -BNP.

Fig. 2 is a chromatogram obtained in an  $\alpha$ -BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample different from that shown in Fig. 1. In Fig. 2, A indicates the position of elution of  $\alpha$ -BNP.

Fig. 3 is a chromatogram obtained in an immunoassay specific for  $\gamma$ -BNP wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample same as those shown in Fig. 2. In Fig. 3, A indicates the position of elution of  $\alpha$ -BNP.

Fig. 4 is a graph showing the relationships between the storing time and BNP immunoreactivity of  $\gamma$ -BNP kept in human plasma at 25°C.

Fig. 5 is a graph showing the relationships between the storing time and BNP immunoreactivity of  $\alpha$ -BNP kept in human plasma at 4°C.

#### BEST MODE FOR CARRYING OUT THE PRESENT INVENTION

[0017] In one embodiment of the present invention, it is related to a method which uses two antibodies, wherein the first antibody is reactive with mammalian  $\alpha$ -BNP and the second antibody is reactive with prepro-BNP or  $\gamma$ -BNP derivatives and is not reactive with  $\alpha$ -BNP.

[0018] Antibodies used in the present method can be monoclonal or polyclonal antibodies. The first antibody can be prepared according to a method known in the art using as an antigen human  $\alpha$ -BNP which is commercially available or

chemically synthesized, or a partial peptide thereof. Alternatively, a monoclonal antibody appended to a commercially available  $\alpha$ -BNP assay system (kit) for measuring  $\alpha$ -BNP ("SHIONORIA", Shionogi) is also available, which is reactive with the C-terminal region of  $\alpha$ -BNP.

[0019] As the second antibody, any antibody can be used subject that it meets the conditions above. Preferred examples of such antibody include those specific for the amino acid sequence shown by the amino acid Nos. 27-102 of SEQ ID NO: 1 or metabolites thereof.

The  $\gamma$ -BNP derivatives as an analyte to be measured by the present method preferably include at least the partial amino acid sequence shown by the amino acid Nos. 27-134 of SEQ ID NO: 1, in case of human BNP. Accordingly, in a preferred embodiment of the present invention, a special attention is preferably paid in the selection of an antigen to obtain an antibody capable of recognizing the amino acid sequence shown by amino acid Nos. 27-102. The preparation of such an antibody can be carried out by any one of methods known in the art.

Theoretically,  $\gamma$ -BNP molecule can be cleaved by protease at sites corresponding to No. 47 (Arg), No. 53 (Lys) and No. 72 (Arg) in the amino acid sequence of SEQ ID NO: 1, and, therefore, an antibody recognizing an amino acid sequence shown by amino acid Nos. 73-102 of SEQ ID NO: 1 can be used as the second antibody.

[0020] The assay of the present invention can be either a competitive- or sandwich-assay and an antibody to be used may be a monoclonal- or polyclonal-antibody.

[0021] At least one of the first and the second antibodies may be labeled detectably or immobilized on a solid support.

[0022] The method for labeling or immobilizing an antibody is known to one ordinary skilled in the art. Examples of label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, and particles. The labeling of an antibody can be carried out according to a method known to one ordinary skilled in the art, for example, that described by Kono et al. (Kaku-Igaku Gijutu, 13(1), 2, (1993)).

[0023] The present invention further provides a kit for immunoassay specific to mammalian  $\gamma$ -BNP derivatives, characterized in that it comprises two antibodies wherein the first antibody is reactive with mammalian  $\alpha$ -BNP and the second antibody is reactive with mammalian prepro-BNP or  $\gamma$ -BNP derivatives and is not reactive with  $\alpha$ -BNP.

[0024] The kit of the present invention can be for a competitive- or sandwich-assay and an antibody to be used may be a monoclonal- or polyclonal antibody.

[0025] At least one of the first and the second antibodies may be labeled detectably or immobilized on a solid support. The kit of the present invention may further contain a means for detecting the label.

[0026] Examples of label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, or particles.

[0027] The following examples and test examples are provided to further illustrate the present invention, without limiting the scope thereof.

#### Example 1

##### Measurement of $\gamma$ -BNP Derivatives by Sandwich IRMA

[0028] Throughout the following Examples, the ordinary reagents used are of special grade supplied by Wako Pure Chemicals Industries, Ltd. or Nacal Tesque, Inc. The bovine serum albumin (BSA) was purchased from Sigma.

#### (1) Preparation of Plasma Sample

##### [0029]

1) Venous blood was collected from patients of cardiac disease or healthy volunteers and placed in blood-collecting tubes containing EDTA and aprotinin (500 KIU/l, Sigma) derived from bovine lung. The tubes were centrifuged ( $\times 2000$  g at  $4^{\circ}\text{C}$ ) for 5 minutes with H-107RGA (Kokusan) to separate blood cells. The resultant plasma samples were frozen and stored at  $-80^{\circ}\text{C}$  until use.

2) The plasma samples prepared in 1) above from patients of cardiac disease or healthy volunteers were fractionated by gel filtration HPLC system LC10A (Shimadzu) equipped with Superdex 75 10/30 column (Pharmacia). After equilibrating the column with 0.1 M phosphate buffer (pH 7.5, 0.3M NaCl, 5 mM EDTA) at flow rate of 1 ml/min, 1 ml of plasma sample was injected and 1 ml each of effluent eluted from the column was collected. Each fraction was subjected to the measurement by assay systems for measuring  $\alpha$ -BNP or  $\gamma$ -BNP as described in (2)-2) and (2)-3) below, respectively.

#### (2) Construction of Assay System For Measuring $\alpha$ -BNP- or $\gamma$ -BNP Derivative

##### [0030]

1) In the assay system, the following peptides, antibodies and kits were used.

- Human  $\alpha$ -BNP (Peptide Institute)
- Antibody against the amino terminal region of  $\gamma$ -hBNP (amino acid Nos. 27-64 of SEQ ID NO: 1) (Peptide Institute)
- Monoclonal antibody against the carboxy terminal structure of  $\alpha$ -BNP (BC203). BC203 is an immobilized antibody appended to SHIONORIA BNP kit (Shionogi), wherein a monoclonal antibody directed to the carboxy terminal structure of  $\alpha$ -BNP is immobilized on beads.
- Monoclonal antibody against the ring structure of  $\alpha$ -BNP (KYBNPII). KYBNPII is a monoclonal antibody appended to the SHIONORIA BNP kit (Shionogi), which is directed to the ring structure (112-128) of  $\alpha$ -BNP, and is labeled with  $^{125}$ I.

## 2) Measurement of Plasma Fraction by Assay System for $\alpha$ -BNP

The measurement of  $\alpha$ -BNP was carried out by commercially available "SHIONORIA BNP kit" (Shionogi). The assay is based on sandwich IRMA (Immunoradiometric Assay) which uses a monoclonal antibody KYBNPII specific for the ring-structure of  $\alpha$ -BNP and another monoclonal antibody BC203 specific for the carboxy terminal structure of  $\alpha$ -BNP. The assay was carried out in accordance with the supplier's instructions.

That is, 100  $\mu$ l each of samples to be assayed or standard solutions (0, 4, 10, 150, 600 or 2000 pg/ml of  $\alpha$ -BNP solution) were dispensed into a polystyrene test tube. To the test tube was added 200  $\mu$ l of iodine-labeled anti-BNP antibody ( $^{125}$ I) solution, followed by one polystyrene bead on which anti-BC203 antibody has been immobilized. The mixture was stirred and allowed to react by leaving stand for 18 hours at 4°C. After washing twice with 2 ml of washing solution, radioactivity was measured on  $\gamma$ -counter ARC-600 (Aloka).

The results are shown in Figs. 1 and 2.

## 3) Measurement of Plasma Fraction by Assay System For $\gamma$ -BNP Derivative

An antibody against amino terminal portion (Nos. 27-64) of  $\gamma$ -hBNP was first labeled with  $^{125}$ I.

IgG was purified from anti-serum (Peptide Institute) raised against amino terminal portion (amino acid Nos. 27-64 of SEQ ID NO: 1) of  $\gamma$ -hBNP using MASPII kit (Bio-Rad) and displaced with 0.5 M phosphate buffer (pH 7.5) using Centricon 30 (Amicon). The labeling of antibody was carried out by the chloramine T method. To a glass tube was dispensed 170  $\mu$ l of purified IgG solution (77.6  $\mu$ g, IgG), and 10  $\mu$ l of Na $^{125}$ I solution (34.2 MBq, Amersham) was added. After addition of 0.1 % chloramine T solution (20  $\mu$ l), the mixture was vigorously stirred at room temperature for 30 seconds. The reaction was quenched by adding 20  $\mu$ l of 0.25 % sodium pyrosulfite solution and 20  $\mu$ l of 5% aqueous potassium iodide solution. When the reaction mixture was treated with Ampure SA column (Amersham) to remove unreacted  $^{125}$ I and to desalt, solution containing  $^{125}$ I-labeled antibody was obtained.

The sandwich IRMA was then carried out in plasma fractions by using the resultant antibody and polystyrene beads on which an antibody recognizing the carboxy terminal structure of  $\alpha$ -BNP (BC203).

To a polystyrene tube was placed 100  $\mu$ l each of samples to be assayed, followed by 200  $\mu$ l of 0.1 M phosphate buffer (pH 7.5, 0.3M, 5 mM EDTA, 0.2% BSA and 500 KIU/l bovine lung aprotinin (Sigma)) and one polystyrene bead on which BC203 antibody has been immobilized. The mixture was stirred and allowed to react by leaving stand for 18 hours at 4°C. After washing twice with 2 ml of washing solution, 300  $\mu$ l of  $^{125}$ I-labeled antibody solution was added. The mixture was stirred and allowed to react by leaving stand for 18 hours at 4°C. After washing twice with 2 ml of washing solution, radioactivity was measured on  $\gamma$ -counter ARC-600 (Aloka). The results are shown in Fig. 3.

## (3) Results

[0031] Figs. 1, 2 and 3 show the chromatograms of gel filtration HPLC of plasma samples obtained from patients, wherein A is the position of elution of  $\alpha$ -BNP.

[0032] Fig. 1 shows the result of the measurement conducted by the  $\alpha$ -BNP assay kit described in (2-2) above. In the Fig. 1, the vertical axis represents the concentration of BNP-like substances in each fraction and the horizontal axis the volume of effluent eluted from the column as measured by SHIONORIA BNP kit. The solid triangle, open square, and open rhombus respectively represent the measurements in different plasma samples.

[0033] Fig. 2 shows the result of the measurement conducted by the  $\alpha$ -BNP assay kit described in (2-2) above in samples different from those shown in Fig. 1. In the Fig. 2, the vertical axis represents the concentration of BNP-like substances in each fraction and the horizontal axis the volume of effluent eluted from the column as measured by SHIONORIA BNP kit. The solid triangle and solid square respectively represent the measurements in different plasma samples.

[0034] From Figs. 1 and 2, it is revealed that there exist substances of molecular weight larger than  $\alpha$ -BNP and having BNP-like immunoreactivity in the plasma of patients of cardiac disease, and that they are the major substances having BNP immunoreactivity.

[0035] Fig. 3 shows the results of the measurement conducted by  $\gamma$ -BNP assay kit described in (2-2) above in the

same samples those shown in Fig. 2. In Fig. 3, the vertical axis represents the radioactivity measured by the  $\gamma$ -BNP immunoassay system and the horizontal axis the volume of effluent eluted from the column. The solid circle represents the measurements of  $\alpha$ -BNP, which obtained after fractionating human  $\alpha$ -BNP solution by HPLC in a similar manner as described in the case of plasma.

[0036] From Fig. 3, it is revealed that the immunoassay specific for  $\gamma$ -BNP derivative of the present invention can detect the major substances with BNP immunoreactivity, but cannot  $\alpha$ -BNP at all.

[0037] The results above indicate that the immunoassay for  $\gamma$ -BNP of the present invention is insensitive to  $\alpha$ -BNP but specific to  $\gamma$ -BNP derivatives. Further, it has also been revealed that  $\gamma$ -BNP is the major substance having BNP immunoreactivity.

#### Test Example 1

##### Stability of $\gamma$ -BNP Derivatives and $\alpha$ -BNP in Plasma

[0038] Fractions suspected to contain  $\gamma$ -BNP derivative were collected from those obtained by treating plasma samples collected from patients of cardiac disease by gel filtration HPLC. Venous blood was collected from healthy volunteers using blood-collecting tubes containing EDTA in the absence of bovine lung aprotinin. Plasma samples (the minimum detection limit of  $\alpha$ -BNP < 4 pg/ml) were prepared in a manner similar to that described in (1)-1) above. The plasma sample was allowed to stand for 0, 2, 6, 24 hours at room temperature (25°C) after addition of the fraction. The stability of BNP derivative was evaluated by determining the BNP immunoreactivity in the plasma sample by means of SHIONORIA BNP kit for assaying  $\alpha$ -BNP.

[0039] Separately, the stability of  $\alpha$ -BNP was evaluated using a plasma sample prepared by adding chemically synthesized  $\alpha$ -BNP to plasma collected from healthy volunteers and standing for 0, 2, 6 and 24 hours at 4°C in the absence of bovine lung aprotinin as described above. The BNP immunoreactivity in the plasma sample was determined by SHIONORIA BNP kit in the same manner as above.

[0040] The stability of  $\gamma$ -BNP derivatives and  $\alpha$ -BNP in plasma samples are shown in Figs. 4 and 5, respectively.

[0041] From Fig. 4, it is revealed that  $\gamma$ -BNP derivatives do not lose significantly the immunoreactivity compared with the initial activity even after 24-hour-standing at 25°C. From Fig. 5, by contrast, it is revealed that  $\alpha$ -BNP loses the immunoreactivity to about 40% based on the initial activity after 24-hour-standing at 4°C.

[0042] The above results demonstrate that  $\alpha$ -BNP is far less stable compared with  $\gamma$ -BNP derivative in blood and that the latter is much more suited in the diagnosis of cardiac diseases than the former.

##### INDUSTRIAL APPLICABILITY

[0043] As mentioned above, the BNP level in patients of heart failure sometimes increases to several tens times to several hundreds times of that of healthy normal subjects, and the change of BNP in the cases of heart failure is so marked that no other hormones are incomparable therewith. For this reason, the usefulness of BNP measurement has been proposed.

[0044] The immunoassay of the present invention allows to determine specifically  $\gamma$ -BNP derivatives without measuring  $\alpha$ -BNP.

[0045] Accordingly, the present immunoassay can be a clinically significant means for diagnosis and prognostic monitoring of heart failure, which leads to conclusion/judgment somehow different from those resulted from conventional BNP assay.

[0046] Further, it is herein disclosed for the first time that  $\gamma$ -BNP, which is a target substance to be assayed by the present method, is stable in blood. Therefore, immunoassay of the present invention provides stable and reliable clinical data without being affected by the process of collecting or storing samples, or the time from the collection until measurement. Further, the immunoassay of the present invention does not require any special pretreatments of blood sample and therefore gives clinical data conveniently, thereby contributing to the establishment of highly accurate diagnosis of cardiac diseases.

##### SEQUENCE LISTING

###### [0047]

<110> Shionogi & Co., Ltd.  
<120> Immunoassay for BNP  
<130> 660984  
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EP 1 016 867 B1

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<150> JP 246684/1997

<151> 1997-9-11

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## 25 Claims

1. An immunoassay specific for mammalian  $\gamma$ -BNP derivatives, characterized in that it uses the first antibody reactive with mammalian  $\alpha$ -BNP and the second antibody reactive with mammalian prepro-BNP or  $\gamma$ -BNP derivatives and not  $\alpha$ -BNP.
2. The immunoassay of claim 1, wherein the  $\gamma$ -BNP derivatives comprise the amino acid sequence shown by the amino acid Nos. 27-102 of SEQ ID NO: 1.
3. The immunoassay of claim 1, wherein the second antibody is specific for the amino acid sequence shown by the amino acid Nos. 27-102 of SEQ ID NO: 1.
4. The immunoassay of any one of claims 1 to 3, wherein at least one of the first and the second antibodies is detectably labeled or immobilized.
5. The immunoassay of any one of claims 1 to 3, wherein the detectable label is a radioactive isotope, an enzyme, a fluorescent substance, a luminescent substance, or a particle.
6. A kit for immunoassay specific for mammalian  $\gamma$ -BNP derivatives, characterized in that it comprises the first antibody reactive with mammalian  $\alpha$ -BNP and the second antibody reactive with mammalian prepro-BNP or  $\gamma$ -BNP derivatives and not  $\alpha$ -BNP.
7. The kit of claim 6, wherein at least one of the first and the second antibodies is detectably labeled or immobilized.
8. The kit of claim 7 which further comprises a means for detecting the label.

## Patentsprüche

1. Immuntest, der spezifisch ist für Säugetier- $\gamma$ -BNP-Derivate, dadurch gekennzeichnet, daß ein erster Antikörper, der mit Säugetier- $\alpha$ -BNP reaktiv ist, und ein zweiter Antikörper, der mit Säugetier-Prepro-BNP- oder  $\gamma$ -BNP-Derivaten und nicht  $\alpha$ -BNP reaktiv ist, verwendet werden.
2. Immuntest gemäß Anspruch 1, worin die  $\gamma$ -BNP-Derivate die durch die Aminosäuren der Nrn. 27-102 der SEQ ID

NO: 1 gezeigte Aminosäuresequenz umfassen.

3. Immuntest gemäß Anspruch 1, worin der zweite Antikörper spezifisch ist für die durch die Aminosäuren der Nrn. 27-102 der SEQ ID NO: 1 gezeigte Aminosäuresequenz.
4. Immuntest gemäß einem der Ansprüche 1 bis 3, worin wenigstens einer der ersten und der zweiten Antikörper mit einer nachweisbaren Markierung versehen oder immobilisiert ist.
5. Immuntest gemäß einem der Ansprüche 1 bis 3, worin die nachweisbare Markierung ein radioaktives Isotop, ein Enzym, eine fluoreszierende Substanz, eine lumineszente Substanz oder ein Partikel ist.
6. Kit für einen Immuntest, der spezifisch ist für Säugetier- $\gamma$ -BNP-Derivate, dadurch gekennzeichnet, daß ein erster Antikörper, der mit Säugetier- $\alpha$ -BNP reaktiv ist, und ein zweiter Antikörper, der mit Säugetier-Präpro-BNP- oder  $\gamma$ -BNP-Derivaten und nicht  $\alpha$ -BNP reaktiv ist, verwendet werden.
7. Kit gemäß Anspruch 6, worin wenigstens einer der ersten und der zweiten Antikörper mit einer nachweisbaren Markierung versehen oder immobilisiert ist.
8. Kit gemäß Anspruch 7, der des weiteren ein Mittel zum Nachweis der Markierung umfaßt.

#### Revendications

1. Analyse immunologique spécifique de dérivés de  $\gamma$ -BNP de mammifères, caractérisée en ce qu'elle utilise le premier anticorps réactif avec un  $\alpha$ -BNP de mammifère et le second anticorps réactif avec un prépro-BNP ou des dérivés de  $\gamma$ -BNP de mammifères et non avec l' $\alpha$ -BNP.
2. Analyse immunologique suivant la revendication 1, dans laquelle les dérivés de  $\gamma$ -BNP comprennent la séquence d'acides aminés représentée par les acides aminés N° 27 à 102 de la SEQ ID N°1.
3. Analyse immunologique suivant la revendication 1, dans laquelle le second anticorps est spécifique de la séquence d'acides aminés représentée par les acides aminés N° 27 à 102 de la SEQ ID N°1.
4. Analyse immunologique suivant l'une quelconque des revendications 1 à 3, dans laquelle au moins l'un des premier et second anticorps est marqué de manière détectable ou immobilisé.
5. Analyse immunologique suivant l'une quelconque des revendications 1 à 3, dans laquelle le marqueur détectable est un isotope radioactif, une enzyme, une substance fluorescente, une substance luminescente ou une particule.
6. Kit pour une analyse immunologique spécifique de dérivés de  $\gamma$ -BNP de mammifères, caractérisé en ce qu'il comprend le premier anticorps réactif avec un  $\alpha$ -BNP de mammifère et le second anticorps réactif avec un prépro-BNP ou des dérivés de  $\gamma$ -BNP de mammifères et non avec l' $\alpha$ -BNP.
7. Kit suivant la revendication 6, dans lequel au moins l'un des premier et second anticorps est marqué de manière détectable ou immobilisé.
8. Kit suivant la revendication 7, qui comprend en outre un moyen pour détecter le marqueur.

Fig. 1

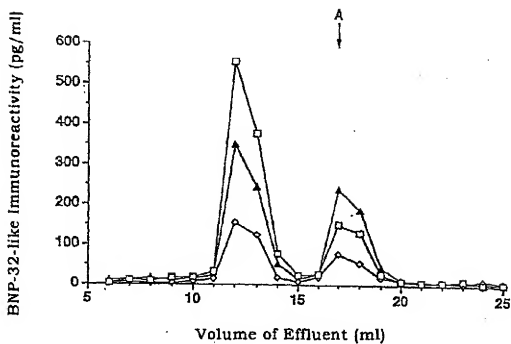


Fig. 2

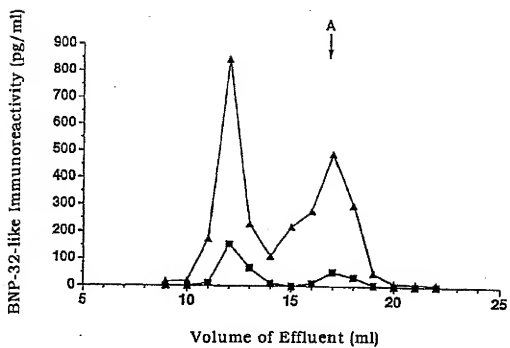


Fig. 3

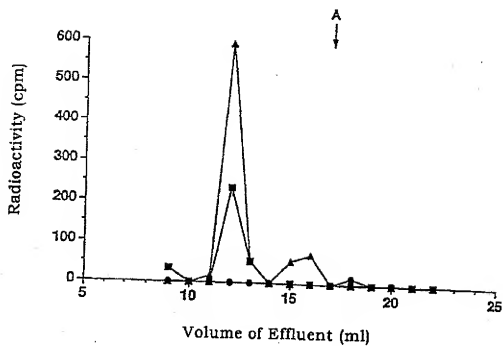


Fig. 4

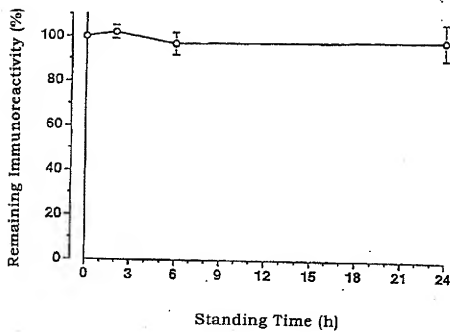


Fig. 5

